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**The effect of the branched-chain amino acids on the *in-vitro* activity of bovine intestinal alkaline
phosphatase**

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Abstract

Branched-chain amino acids (BCAA) are used as nutritional support for patients with a range of conditions including liver cirrhosis and in-born errors of amino acid metabolism, and are commonly used “sports”/exercise supplements. The effects of the BCAA on the *in-vitro* activity of calf intestinal alkaline phosphatase (EC. 3.1.3.1) were studied. All three BCAA were found to be uncompetitive inhibitors of the enzyme with L-leucine being the most potent ($K_i' = 24.9\text{mM}$) and L-valine, the least potent ($K_i' = 37\text{mM}$). Mixed BCAA are able to act in combination to inhibit the enzyme. Given the important role of intestinal alkaline phosphatase in gut homeostasis, these findings have potential implications for those taking high levels of BCAA as supplements.

Keywords; Intestinal Alkaline Phosphatase, Branched-chain amino acids, supplements, L-leucine, L-isoleucine, L-valine.

Introduction

Intestinal alkaline phosphatase (IAP) has been demonstrated to play many important physiological roles (Lalles 2010) and IAP's interactions with the microbiota, the diet and the gut make it central to gut homeostasis (Estaki *et al.* 2014). IAP is involved in the absorption of dietary lipids across the gut wall (Narisawa *et al.* 2003; Eliakim *et al.* 1991; Tietze *et al.* 1992) and there is also evidence that IAP is involved in the regulation of surface pH in the duodenum (Akiba *et al.* 2007). In addition to these functions supporting the digestive activity of the gut, it has been demonstrated that IAP is able to help protect the body from pathogenic bacteria. IAP has been shown to limit bacterial passage from the lumen to the mesenteric lymph nodes (Goldberg *et al.* 2008) and is able to detoxify bacterial lipopolysaccharide (Beumer *et al.* 2003). These studies suggest that the interactions between the gut microbiota and IAP are crucial for intestinal health. It has been demonstrated that dietary factors affect IAP expression and activity; fasting decreases IAP expression/activity and this activity is restored when feeding recommences (Goldberg *et al.* 2008). Similarly, it has been demonstrated that levels of specific macronutrients, notably fats and proteins, in the diet can affect IAP (Alpers *et al.* 1995; Lynes *et al.* 2011; Montoya *et al.* 2006).

As essential amino acids, an adequate intake of BCAA is crucial to maintaining general health and supplementation with BCAA has been investigated in various clinical settings (Holecek 2018), including the treatment of cachexia in cancer patients (Choudry *et al.* 2006; Chen *et al.* 2015) and as nutritional support for patients with BCAA deficiencies (Joint Formulary Committee 2017-2018).

Protein and essential amino acid supplementation is also a common activity in sports/fitness settings. As part of this regime, BCAA supplementation has become commonplace and there has been much research on the possible ergogenic effects of leucine (Witard *et al.* 2016; Martin *et al.* 2017). Supplementation with

leucine has a direct effect on the activation of the mammalian target of rapamycin (mTOR) pathway and the stimulation of muscle protein synthesis (MPS) (Foster and Fingar 2010; Reidy and Rasmussen 2016). These effects on MPS have been suggested to be dependent upon a number of factors such as nutrient timing strategies (Dickinson *et al.* 2014) and optimal dose (Churchward-Venne *et al.* 2014; Glynn *et al.* 2010; Mero 1999).

L-Leucine (L-Leu) has previously been reported to be an uncompetitive inhibitor of human AP (Hummer and Millan 1991; Hoylaerts *et al.* 1992). Given the important role of IAP in intestinal homeostasis and general health and the use of BCAA supplements, the present study sought to investigate the effects of the other BCAA and their combined effects on the *in-vitro* activity of IAP, using commercially available enzyme purified from calf intestine, as a model system.

Materials and methods

Unless otherwise stated all materials were obtained from Sigma-Aldrich UK Ltd and were the highest grades available. Enzyme was prepared in bulk at 1mg/ml in 0.1M Glycine-NaOH buffer, pH9.8, 1mM MgCl_2 and stored at -20°C until use.

The activity of the enzyme was measured by following the hydrolysis of p-nitrophenol phosphate (pNPP) to p-nitrophenol (pNP) at 400nm using a Jenway 7315 spectrophotometer. 0.068M Glycine-NaOH buffer, pH9.8, 0.68mM MgCl_2 was used and the velocity of the reaction at substrate (pNPP) concentrations ranging from 0.024-1.2mM were studied. Enzyme concentration in each assay was 0.08mg/ml (≥ 10 units/mg). The effects of the BCAA on the enzyme were examined by repeating the measurements of enzyme velocity in the presence of BCAA concentrations ranging from 0-20mM. All assays were performed in triplicate at $21 \pm 1^\circ\text{C}$. The initial linear rate of the reaction was determined by linear regression of the experimental data and comparison to a pNP solution of known concentration (2.875×10^{-5} M). The K_m and V_{\max} values of the enzyme were obtained by fitting the substrate concentration v velocity data to the Michaelis curve by a non-linear least squares method. The K_m and V_{\max} values in the different experimental groups were analysed using a one-way ANOVA (not assuming equal variance). Where statistically significant differences were found, pairwise Welch's t-Tests using the Holm-Bonferroni correction were used poc hoc. The K_i' value of each inhibitor was determined using a secondary plot of inhibitor concentration v the reciprocal of the observed V_{\max} value, the x-axis intercept of this plot being equal to $-K_i'$. All data and statistical analyses were performed using "R" version 3.3.2 (R Core Team 2016).

Results

Effect of L-leucine on calf-IAP

L-leucine decreased the K_m of calf-IAP in a concentration dependent manner at concentrations ranging from 1-10mM (see Figure 1a). One-way ANOVA suggested a statistically significant difference between groups ($F_{(3,15,13)} = 39.139$, $p=2.283 \times 10^{-7}$). Pairwise t-Tests using the Holm-Bonferroni correction established significant differences between all groups ($p<0.05$).

Similarly, L-leucine also decreased the V_{max} of calf-IAP (see Figure 1b) and one-way ANOVA suggested statistically significant differences between the groups ($F_{(3,15,135)} = 17.195$, $p = 3.6 \times 10^{-5}$). Pairwise t-Tests using the Holm-Bonferroni correction suggested that this effect was only statistically significant at concentrations above 1mM L-leucine and that the difference between adjacent concentrations of the L-leucine was not statistically significant.

The K_i' of L-leucine was calculated to be 24.9mM.

Effect of L-Isoleucine on calf-IAP

As was the case with L-leucine, L-isoleucine decreased the K_m of calf-IAP in a concentration dependent manner (see Figure 2a). One-way ANOVA suggested a statistically significant difference between groups

($F_{(3, 10.38)} = 86.524$, $p = 1.22 \times 10^{-7}$). Pairwise t-Tests using the Holm-Bonferroni correction established significant differences between all groups ($p < 0.05$).

L-isoleucine also had a statistically significant effect on the V_{\max} of the enzyme (see Figure 2b), ($F_{(3, 10.99)} = 7.395$, $p = 0.0055$). Pairwise t-Tests using the Holm-Bonferroni correction suggested that this effect was only statistically significant at concentrations above 1mM L-isoleucine and that the differences observed between the different treatment groups were not statistically significant.

The K_i' of L-Isoleucine was calculated to be 35.1mM.

Effect of L-valine on calf-IAP

In agreement with the other members of the BCAA, L-valine decreased the K_m of the enzyme (see Figure 3a). One-way ANOVA suggested statistically significant differences between the groups ($F_{(3, 14.96)} = 51.05$, $p = 4.3 \times 10^{-8}$). Pairwise t-Tests using the Holm-Bonferroni correction established significant differences between all groups ($p < 0.05$).

L-valine also had a statistically significant effect on the V_{\max} (see Figure 3b), $F_{(3, 15.51)} = 14.513$, $p = 9.05 \times 10^{-5}$). Pairwise t-Tests using the Holm-Bonferroni correction suggested that this effect was only statistically significant at concentrations above 1mM L-valine.

The K_i' of L-valine was calculated to be 37mM.

Effect of mixed BCAAs on calf-IAP

Those who take BCAA supplements often do so as mixtures, most commonly in the ratio of 2:1:1, (L-leucine: L-isoleucine: L-valine). The effects of such a mixture of BCAA was examined in the present study, total BCAA concentrations ranged from 2-20mM. As observed with the individual amino acids, the BCAA

mixture decreased the K_m of the enzyme (see Figure 4a), ($F_{(3, 10.09)} = 175.56$, $p = 5.27 \times 10^{-9}$). Pairwise t-Tests using the Holm-Bonferroni correction established significant differences between all groups ($p < 0.05$).

Similarly, the BCAA mixture decreased the V_{max} of the enzyme (see Figure 4b), ($F_{(3, 10.89)} = 58.14$, $p = 5.39 \times 10^{-7}$). Pairwise t-Tests using the Holm-Bonferroni correction established significant differences between all groups ($p < 0.05$).

The K_i' of the BCAA mixture was calculated to be 22.1mM.

Discussion

There has been a longstanding clinical interest in the use of BCAA supplements to support patients with a range of conditions (Holecek 2018) and the BCAA are licensed in the UK for use as nutritional support in a number of clinical situations (Joint Formulary Committee 2017-18). The use of these supplements particularly in a “sports”/fitness context has also shown considerable growth in recent years (Close *et al.* 2016). The branched chain amino acids; L-leucine, L-isoleucine and L-valine are popular supplements reputed to stimulate MPS (Jackman *et al.* 2017), preserve lean muscle mass during weight loss strategies (Dudgeon *et al.* 2016), and to augment recovery after resistance training (Howatson *et al.* 2012). The current study sought to determine the *in-vitro* effects of these substances on IAP. The enzyme used in this study was the readily available calf-IAP but this is also a good model system for the human isozyme. Sequence alignment between human (NP_001622.2)(Kiffer-Moreira *et al.* 2014) and bovine (AAA30571.1)(Weissig *et al.* 1993) IAP suggested 77% homogeneity between the proteins. In addition to this global similarity, the residues identified as being essential to AP activity in the human placental and

germ-cell forms are conserved in the bovine intestinal isozyme (Kozlenkov *et al.* 2002). Moreover, the K_m values observed in the present study were entirely consistent with previously reported values for human APs acting on p-NPP (Hoylaerts *et al.* 1992; Hummer and Millan 1991).

The effects of the BCAA on the kinetic parameters of calf-IAP were examined at concentrations between 1-10mM. We found that L-leucine produced a dose dependent decrease in both the K_m (fig 1a) and V_{max} (fig 1b) of the enzyme, only concentrations of L-leucine in excess of 1mM produced a statistically significant decrease in the V_{max} . These results suggest that L-leucine is an uncompetitive inhibitor of calf-IAP with a K_i' of around 25mM and are in keeping with previous reports of the effect of L-leucine on AP (Hummer and Millan 1991; Hoylaerts *et al.* 1992). The effects of the other BCAA have not previously been reported; L-isoleucine also produced a dose dependent decrease in the K_m (fig 2a) and V_{max} (fig 2b) of the enzyme suggesting that it is also an uncompetitive inhibitor, though rather less potent (the enzyme has a lower affinity for L-isoleucine) than L-leucine with a K_i' of 35mM. Similarly L-valine was found to decrease both the K_m (fig 3a) and V_{max} (fig 3b) of the enzyme and so would also appear to be an uncompetitive inhibitor with a K_i' (37mM) similar to that of L-isoleucine. Given the structural similarity of the BCAA it would seem likely that they are binding to the same site and that this site is relatively specific for L-leucine. The use of a mixture of all three amino acids was studied, mimicking the situation where the BCAA are consumed in the ratio, 2:1:1. Total BCAA concentrations studied were 2-20mM, again this produced a concentration dependent decrease in both the K_m (fig 4a) and V_{max} (fig 4b) of the enzyme. In the case of the individual amino acids the lowest concentration studied (1mM) did not produce a statistically significant decrease in the V_{max} of the enzyme (figs 1b, 2b and 3b) but the higher concentrations did. When studying the mixture of all three amino acids the lowest concentration used was 2mM and this did produce a statistically significant decrease in the V_{max} , suggesting that the different BCAA may be able to act in combination to inhibit IAP. It seems likely that all BCAA bind to the same inhibitor site on the enzyme and that the L-isoleucine and L-valine may contribute to the inhibition mediated by the L-leucine. This is

supported by the finding that the K_i' for the BCAA mixture was 22mM, similar to the K_i' of leucine alone (24.9mM). In the present study, the inhibitory effects of the BCAA on IAP appears to have an upper limit. In all legs of the study, the maximum inhibition observed was around 30-40%, regardless of the BCAA concentration ranges used (1-10mM or 2-20mM). Sports/fitness supplementation in humans could produce higher concentrations e.g., a 2g dose taken with 100ml of H₂O on an empty stomach would produce a gastric concentration of ~105mM. It is possible that such higher BCAA concentrations could produce greater inhibition of the enzyme as observed in previous studies of the effects of L-leucine on APs (Hoylaerts *et al.* 1992; Hummer and Millan 1991). However, this seems unlikely since the data from the present study suggest that the effects of the inhibitors are plateauing at the upper end of the concentration ranges used. This difference could reflect the fact that the intestinal form of AP is less sensitive to the inhibitory effects of the BCAA (K_i' for L-leucine of 24.9mM) than the placental (K_i' =9.2mM) or germ-cell forms (K_i' =0.54mM) used in the earlier studies (Hoylaerts *et al.* 1992).

Conclusion

All three of the branched-chain amino acids (BCAA) are uncompetitive inhibitors of calf-IAP. L-leucine was the most potent inhibitor studied ($K_i' = 24.9\text{mM}$) with L-isoleucine and L-valine being noticeably less effective ($K_i' = 35$ and 37mM respectively). Studies using a mixture of all three BCAA suggested that the amino acids were able to combine to inhibit the enzyme. Given the importance of IAP in gut homeostasis, these results have potential implications for those consuming high levels of BCAA as “sports”/fitness supplements.

Conflicts of interest

The authors have no conflicts of interest to report.

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Figure captions

Fig 1. The effect of L-leucine on intestinal alkaline phosphatase. The *in-vitro* activity of calf-IAP was measured in the presence of increasing concentrations of L-leucine (0-10mM) and the effects on the K_m (A) and V_{max} (B) of the enzyme were determined (n=8). Data presented as standard boxplots. Pairwise t-tests using the Holm correction were used to determine statistically significant differences between the group means.

Fig 2. The effect of L-isoleucine on intestinal alkaline phosphatase. The *in-vitro* activity of calf-IAP was measured in the presence of increasing concentrations of L-Isoleucine (0-10mM) and the effects on the K_m (A) and V_{max} (B) of the enzyme were determined (n=6). Data presented as standard boxplots. Pairwise t-tests using the Holm correction were used to determine statistically significant differences between the group means.

Fig 3. The effect of L-valine on intestinal alkaline phosphatase. The *in-vitro* activity of calf-IAP was measured in the presence of increasing concentrations of L-valine (0-10mM) and the effects on the K_m (A) and V_{max} (B) of the enzyme were determined (n=8). Data presented as standard boxplots. Pairwise t-tests using the Holm correction were used to determine statistically significant differences between the group means.

Fig 4. The effect of mixed BCAA on intestinal alkaline phosphatase. The *in-vitro* activity of calf-IAP was measured in the presence of increasing concentrations of mixed BCAA (L-leu: L-isoleu: L-val; 2:1:1) (0-20mM) and the effects on the K_m (A) and V_{max} (B) of the enzyme were determined (n=6). Data presented as standard boxplots. Pairwise t-tests using the Holm correction were used to determine statistically significant differences between the group means.

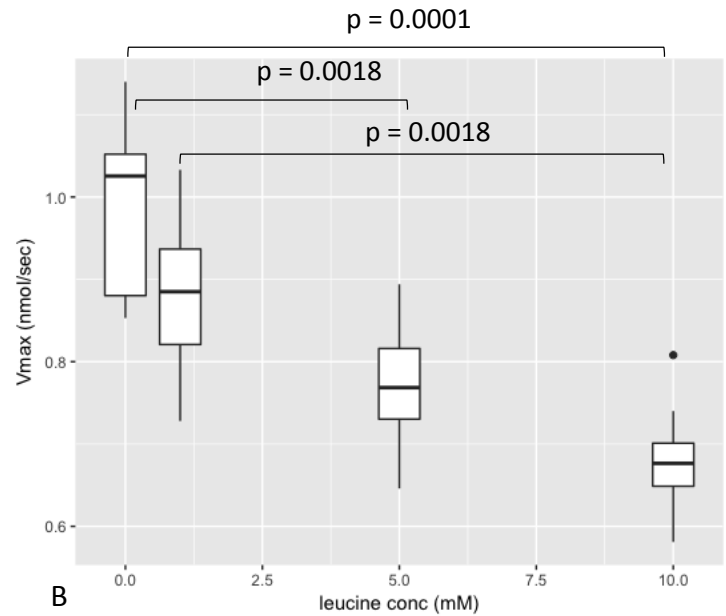
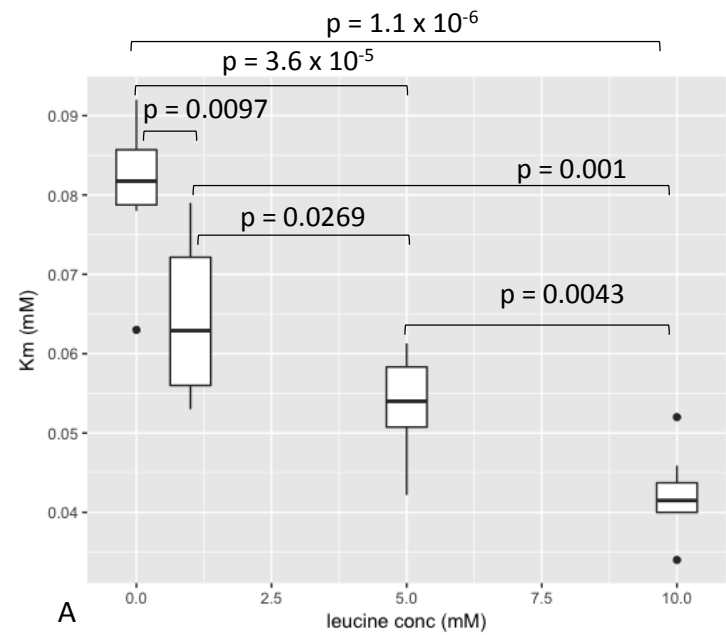


Fig. 1

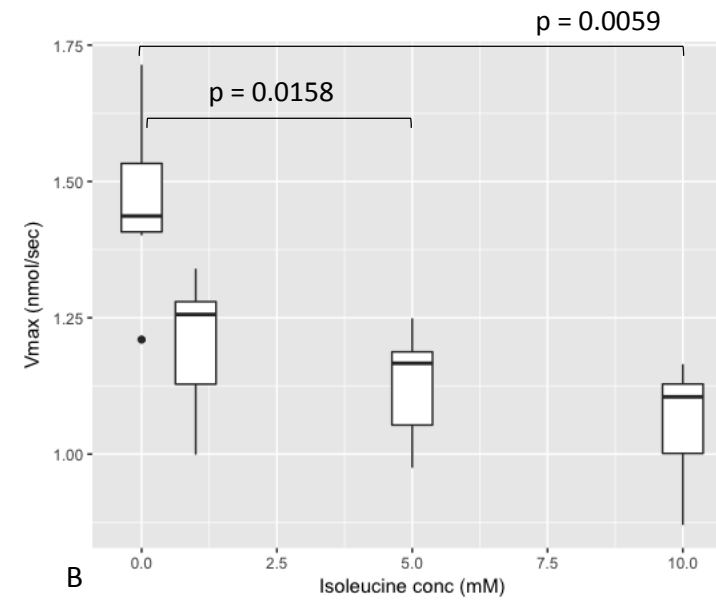
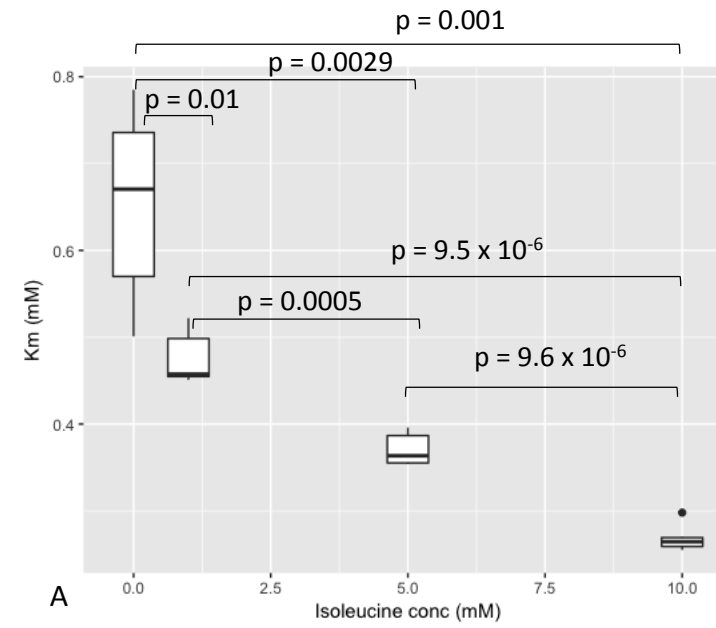


Fig. 2

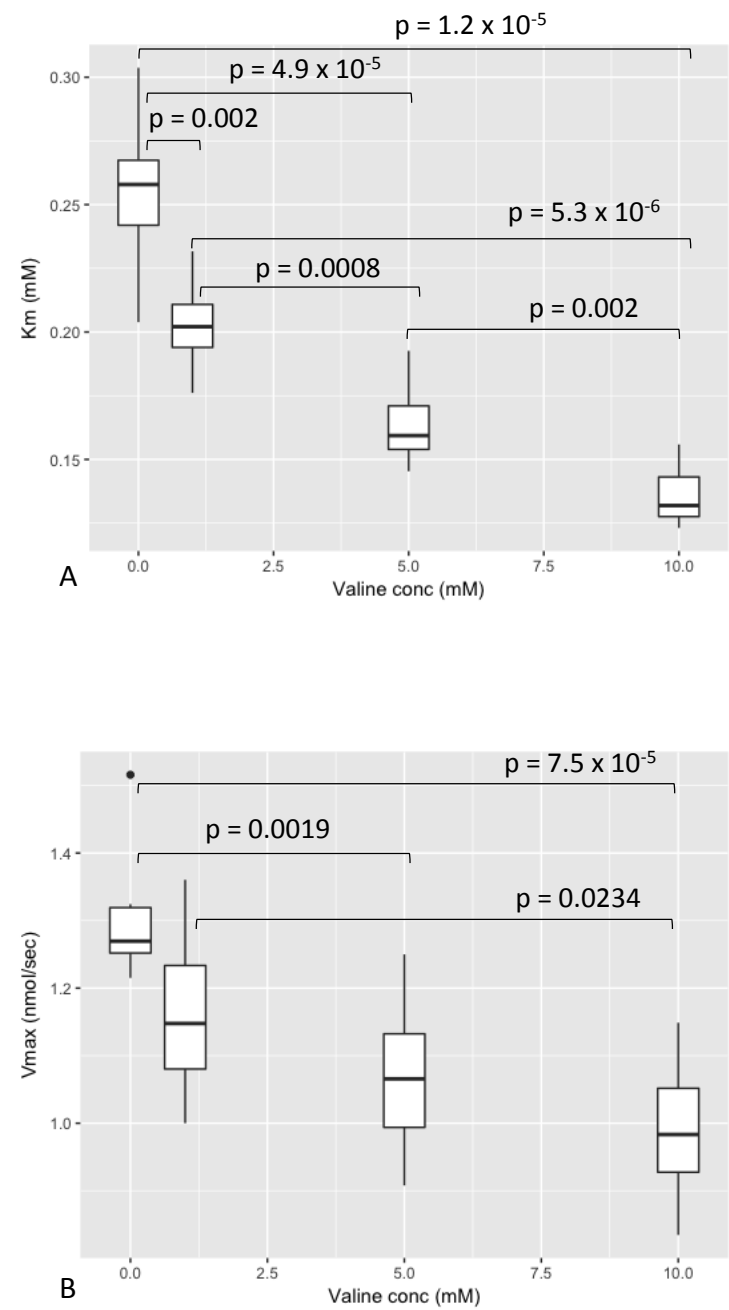


Fig. 3

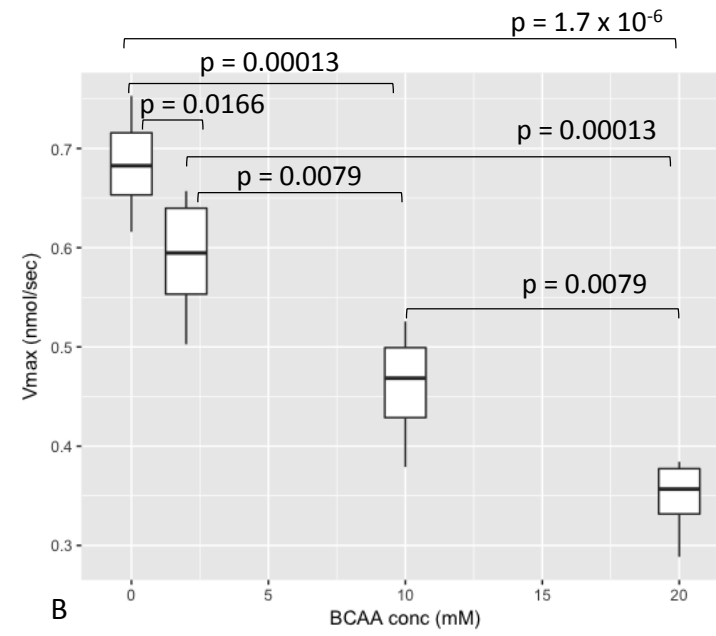
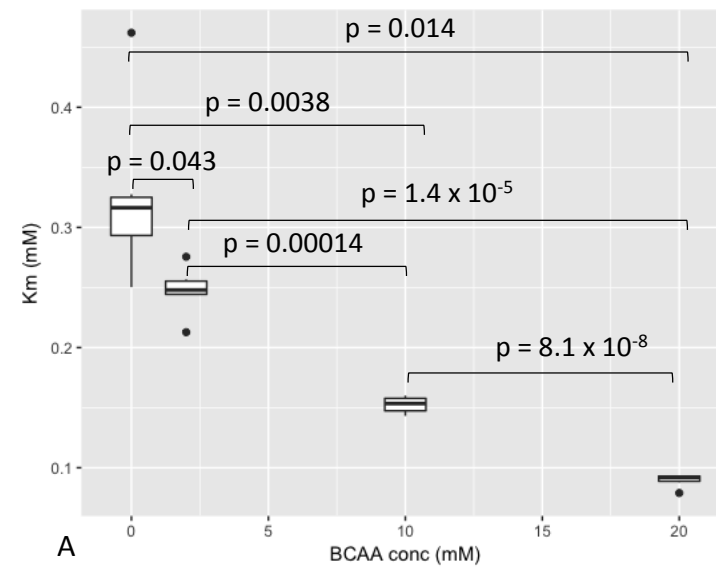


Fig.4